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Development of a New, Combined Rapid Method Using Phage and PCR for Detection and Identification of Viable *Mycobacterium paratuberculosis* Bacteria within 48 Hours▽

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The *FASTPlaque*TB assay is an established diagnostic aid for the rapid detection of *Mycobacterium tuberculosis* from human sputum samples. Using the *FASTPlaque*TB assay reagents, viable *Mycobacterium avium* subsp. *paratuberculosis* cells were detected as phage plaques in just 24 h. The bacteriophage used does not infect *M. avium* subsp. *paratuberculosis* alone, so to add specificity to this assay, a PCR-based identification method was introduced to amplify *M. avium* subsp. *paratuberculosis*-specific sequences from the DNA of the mycobacterial cell detected by the phage. To give further diagnostic information, a multiplex PCR method was developed to allow simultaneous amplification of either *M. avium* subsp. *paratuberculosis* or *M. tuberculosis* complex-specific sequences from plaque samples. Combining the plaque PCR technique with the phage-based detection assay allowed the rapid and specific detection of viable *M. avium* subsp. *paratuberculosis* in milk samples in just 48 h.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of Johne's disease, a degenerative wasting disease that primarily infects ruminants, including domestic livestock, such as cattle, sheep, and goats. In the dairy industry, Johne's disease leads to considerable economic losses due to a decrease in milk production and reduced fertility in infected cattle (16, 17). Due to the possible link between *M. avium* subsp. *paratuberculosis* and the development of Crohn's disease in humans (4, 15), *M. avium* subsp. *paratuberculosis* has also become of interest as a public health issue despite the fact that a causal link between the organism and human disease has not been unequivocally established. A recent survey in the United Kingdom has shown that viable *M. avium* subsp. *paratuberculosis* cells can be cultured from pasteurized retail milk (10), indicating that either *M. avium* subsp. *paratuberculosis* may occasionally survive high-temperature, short-time pasteurization treatments or postprocess contamination is occurring.

Both efforts to manage Johne's disease and studies of heat resistance of *M. avium* subsp. *paratuberculosis* have been hampered by the lack of rapid, specific detection tests for viable *M. avium* subsp. *paratuberculosis* cells. An ideal *M. avium* subsp. *paratuberculosis* detection test would be low cost, rapid, and specific and provide live/dead differentiation. Culture is currently regarded as the definitive method for the detection of viable *M. avium* subsp. *paratuberculosis* bacteria; however, *M. avium* subsp. *paratuberculosis* is an extremely fastidious organism and requires the longest incubation periods of all the mycobacteria cultured to date (6 to 16 weeks). Additionally, harsh chemical decontamination of samples is required to suppress growth of competitive microorganisms, which can reduce the sensitivity of culture to detect *M. avium* subsp. *paratuberculosis* (6). PCR assays have been successfully used to detect the presence of *M. avium* subsp. *paratuberculosis* based on the amplification of the sequences IS*900* (9), F57 (5, 18), and more recently the newly identified multicopy ISMAP*02* sequence (22). However, the PCR assays alone cannot provide live/dead differentiation. Reverse transcription (RT)-PCR assays can overcome this limitation to some extent by amplifying IS*900* mRNA from *M. avium* subsp. *paratuberculosis* cells, demonstrating that gene transcription is taking place (14), but RT-PCR methodology is difficult to apply to the routine testing of bacteria in food samples or other complex sample matrices due to difficulty in extracting cells from the matrix and the carry-through of contaminants that inhibit DNA amplification (11). A variety of immunological tests have been developed that indirectly detect *M. avium* subsp. *paratuberculosis* infection by assaying species-specific cell-mediated or humoral immune responses in the host. Immune response assays are generally rapid and easy to perform and are a useful tool for determining *M. avium* subsp. *paratuberculosis* infection at the herd level. However, these assays are often limited by issues of specificity and the fact that a variable immunological response is seen during different stages of infection (see reference 25 for a review).

The *FASTPlaque*TB assay (FPTB) is an established tool for the rapid detection of *Mycobacterium tuberculosis* and is used in developing countries as an inexpensive, rapid diagnostic aid for the detection of viable *M. tuberculosis* cells in sputum (for

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TABLE 1. Bacteria and insertion sequences

Bacterium or strain	Source	IS element
M. avium subsp. *paratuberculosis* ATCC 19851	Bovine isolate ATCC	IS*900*
M. avium subsp. *paratuberculosis* B4	Bovine isolate, UK[a] I. Grant[b]	IS*900*
M. avium subsp. *paratuberculosis* DVL 943	Bovine isolate, The Netherlands I. Grant[b]	IS*900*
M. avium subsp. *paratuberculosiss* pw 377	Bovine milk, UK I. Grant[b]	IS*900*
M. smegmatis	Biotec Laboratories[c]	
M. bovis BCG NCTC 5692	NCTC	IS*6110* (1 copy) IS*1081* (5–6 copies)
M. tuberculosis H37Rv	M. Barer[d]	IS*6110* (1–20 copies) IS*1081* (5–6 copies)

[a] UK, United Kingdom.
[b] Queens University, Belfast, United Kingdom.
[c] Biotec Laboratories, Ipswich, United Kingdom.
[d] University of Leicester, Leicester, United Kingdom.

a review, see reference 19). This type of assay (termed the phage amplification or PhaB assay) is based on the successful replication of phage to indicate the presence of viable host cells, with the end point of the assay being the formation of plaques. In outline, samples containing the *M. tuberculosis* cells are mixed with phage and are incubated to allow infection. At this point a virucide is added to destroy any phage that have not infected cells, and so only those phage protected by the host cell survive. To detect these protected phage, the sample is mixed with *Mycobacterium smegmatis* and mixed with agar to form a lawn, and plates are incubated overnight. Lysis of the infected target cell releases phage, which then form plaques by infection of the *M. smegmatis* cells. Each plaque represents the presence of a mycobacterial cell capable of being infected by the phage in the original sample, and in human sputum this is most likely to be *M. tuberculosis*. A key part of this assay is the fact that the mycobacteriophage used (D29) has a host range broader than just *M. tuberculosis* and also infects other types of mycobacterial cells, and therefore, we wished to investigate whether the FPTB assay reagents could also be used for the detection of *M. avium* subsp. *paratuberculosis*. To increase the specificity, the phage amplification assay was combined with a PCR-based identification method for molecular confirmation of the cell detected. Here we describe the successful development of this combined phage-PCR method, which gives both live/dead differentiation and molecular characterization of the cell detected, and show that it can be extended for the detection and identification of other mycobacteria from samples containing a mixed flora without the need to remove competitive microflora before detection.

MATERIALS AND METHODS

Bacterial strains and culture media. The mycobacteria strains used in this study are listed in Table 1. *M. avium* subsp. *paratuberculosis* cells were cultured on Herrold's egg yolk medium with mycobactin J (37°C for 12 to 16 weeks). *M. tuberculosis* was cultured on Middlebrook 7H10 oleic acid-albumin-dextrose-catalase agar plates (37°C for 3 to 4 weeks); *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) cells were cultured (37°C for 3 to 4 weeks) on Löwenstein-Jensen slopes with glycerol (MAST Diagnostics). *M. smegmatis* cells were grown in Middlebrook 7H9 oleic acid-albumin-dextrose-catalase broth (50 ml; Difco Laboratories) with shaking (37°C for 48 h at 200 rpm).

Phage detection assays. The FPTB assay reagents were prepared and used according to the manufacturer's instructions (Biotec Laboratories Ltd., Ipswich, United Kingdom). Bacterial samples were prepared by recovering mycobacterial cells from one slope of solid culture medium and directly resuspending these in 1 ml FPTB Media Plus. Actiphage (100 μl containing 10^8 PFU mycobacteriophage) was added to each test sample and incubated (37°C, 1 h) to allow infection. The sample was then thoroughly mixed with Virusol (100 μl; virucidal solution) and incubated (room temperature, 5 min) to inactivate any remaining extracellular phage. Virusol activity was neutralized by addition of FPTB Media Plus (5 ml) and sensor cells (1 ml; 10^8 CFU *M. smegmatis*) and of molten FPTB agar (5 ml, 55°C). Samples were poured into a petri dish and incubated (37°C, 18 h). The presence of mycobacteria in the test sample was indicated by the presence of plaques. Positive and negative assay controls supplied with the kit were included with each analysis, according to the manufacturer's instructions.

Preparation of genomic and plaque DNA. For *M. avium* subsp. *paratuberculosis*, *M. smegmatis*, and *M. bovis* BCG genomic DNA, a loopful (10 μl) of bacteria was resuspended in high-performance-liquid-chromatography-grade sterile water (1 ml), heated (100°C, 20 min), and then centrifuged (13,000 × *g*, 15 min) and the supernatant (5 μl) used for PCR amplification. *M. tuberculosis* H37Rv genomic DNA was a gift of the Department of Microbiology, Immunology and Pathology, Colorado State University. Plaque DNA samples were prepared by excising an agar plug (approximately 10 μl) from the center of a plaque using a sterile polypropylene 1,000-μl micropipette tip. The agar plug was transferred to high-performance-liquid-chromatography-grade sterile water (10 μl), heated (95°C, 5 min), and immediately frozen (−20°C). After a minimum of 15 min at this temperature, samples were thawed and 10 μl of supernatant was used per PCR.

PCR conditions. Primers used for the amplification of signature sequences were P90 and P91 (IS*900* [26]), JB21 and JB22 (500-bp *M. bovis* gene fragment [21]), TB850 and TB284 (IS*6110* [27]), and BW6 and BW7 (IS*1081* [24]). PCRs were carried out in a Techne PCR block (Progene) in a reaction volume of 50 μl using ABgene 10× buffer, 250 μM of each deoxyribonucleotide triphosphate (Promega), 2.5 mM $MgCl_2$, 1 U of *Taq* polymerase (ABgene), and 250 ng primer. For duplex or multiplex PCR, 3 mM $MgCl_2$ was used and each primer was used at 150 ng. Amplification conditions used were initial denaturation at 94°C for 5 min, followed by 37 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 4 min. PCR products were visualized after agarose gel (2% [wt/vol]) electrophoresis by staining with ethidium bromide (0.33 μg ml^{-1}) and UV illumination. PCR products were sequenced by the University of Nottingham, Sutton Bonington Campus sequencing unit.

RESULTS

Detection of *M. avium* subsp. *paratuberculosis* using the FPTB assay. To determine if the FPTB assay could be used to detect *M. avium* subsp. *paratuberculosis*, a cell suspension of *M. avium* subsp. *paratuberculosis* ATCC 19851 was 10-fold serially

TABLE 2. Comparison of the *M. avium* subsp. *paratuberculosis* cell numbers and plaques recorded

Maximum no. (CFU ml^{-1}) of *M. avium* subsp. *paratuberculosis*[a]	No. of plaques (PFU ml^{-1})
1×10^7	Complete lysis[b]
1×10^6	Complete lysis[c]
1×10^5	Confluent lysis[c]
1×10^4	Confluent lysis[c]
1×10^3	TNTC[d]
1×10^2	57
0	0
1×10^7 (heat killed)[e]	0

[a] Cell mass was estimated from the optical density of cell suspensions.
[b] Complete lysis, lysis of all of the lawn of *M. smegmatis* cells, indicating $>10^5$ *M. avium* subsp. *paratuberculosis* cells were detected.
[c] Confluent lysis: lysis of 80 to 90% of the lawn of *M. smegmatis* cells, indicating the detection of 10^4 to 10^5 *M. avium* subsp. *paratuberculosis* cells.
[d] TNTC, too numerous to count (10^3 to 10^4 MAP plaques per plate with some merging of individual plaques).
[e] Heat treated at 100°C for 20 min.

diluted in FPTB Media Plus and samples tested with the FPTB assay. From the optical density of the suspension, it was predicted that approximately 1×10^7 CFU ml^{-1} were present and that numbers in the countable range would be reached at the 10^{-5} dilution. Plaques were detected only at the end point of the assay when *M. avium* subsp. *paratuberculosis* cells were added, indicating that the phage was able to infect *M. avium* subsp. *paratuberculosis* cells, and the number of plaques detected (57 at the 10^{-5} dilution [Table 2]) correlated with the expected number of *M. avium* subsp. *paratuberculosis* cells in the test sample. When samples containing heat-killed *M. avium* subsp. *paratuberculosis* cells (1×10^7 CFU ml^{-1} *M. avium* subsp. *paratuberculosis* ATCC 19851 heat treated at 100°C for 20 min) were tested, no plaques were formed, indicating that the *M. avium* subsp. *paratuberculosis* cells must be viable for detection by the FPTB assay. This finding indicated that the current FPTB assay format and components were suitable for the detection of viable *M. avium* subsp. *paratuberculosis* cells.

PCR from plaques generated by the FPTB *M. avium* subsp. *paratuberculosis* assay. To add specificity to this assay, we investigated whether the mycobacterial cell giving rise to an individual plaque could be identified by PCR. Primers P90 and P91 (26) were chosen to amplify a 400-bp fragment of the multicopy IS*900* gene found in all *M. avium* subsp. *paratuberculosis* strains (Tables 1 and 2). These primers were chosen because the IS*900* method is a well-established identification method for *M. avium* subsp. *paratuberculosis* and because it was more likely that a multicopy gene could be detected in the DNA originating from a single cell. After *M. avium* subsp. *paratuberculosis* cells had been processed through the FPTB assay, DNA was extracted by excising agar from the center of a plaque and heating at 95°C. Samples were then frozen at −20°C for a minimum of 15 min, since this was found to give the most efficient separation of the DNA from the agar. Samples were thawed at room temperature, and the supernatant was used as the template for the PCR.

Four different strains of *M. avium* subsp. *paratuberculosis* were processed through the assay to generate plaques containing *M. avium* subsp. *paratuberculosis* DNA (Fig. 1). A PCR product of 400 bp for the target IS*900* gene was amplified from plaque DNA extracted from all four *M. avium* subsp. *paratuberculosis* strains tested (Fig. 1, lanes 2 to 13). Sequence analysis of the PCR products was used to confirm that the PCR products arose from the specific amplification of the IS*900* gene. The amount of PCR product achieved for the different *M. avium* subsp. *paratuberculosis* strains tested varied and may reflect either variation in the copy number of the IS*900* sequence (generally strains contain between 14 and 18 copies of IS*900*) or the propensity of the *M. avium* subsp. *paratuberculosis* cells to clump (hence plaques may arise from a group of cells rather than a single cell). As expected, the amount of product amplified from DNA extracted from the plaques was generally smaller than that of the control reaction carried out using genomic DNA as the template (Fig. 1, lane 15). As a negative control, *M. smegmatis* cells were processed through the FPTB assay and plaque DNA tested by PCR for the presence of IS*900* sequences. In this case no PCR product was generated (Fig. 1, lane 14).

The finding that the DNA of the original *M. avium* subsp. *paratuberculosis* cell was preserved in the plaque after overnight incubation was somewhat surprising. To rule out the possibility that we were simply detecting uninfected *M. avium* subsp. *paratuberculosis* cells carried through the assay into the agar, *M. avium* subsp. *paratuberculosis* cells were processed through the assay and agar plugs extracted both from plaques and also from the lawn of the same plates in areas where no plaques were present ($n = 18$ for each sample type). DNA was extracted from both types of sample and used as a template for PCR amplification of the IS*900* sequence. The 400 bp-band was amplified from all the plaque DNA samples as expected, and no PCR products were generated from the PCR using agar plugs extracted from the lawn area (data not shown). This finding confirmed that the amplification of the 400-bp region of the IS*900* gene was from the *M. avium* subsp. *paratubercu-*

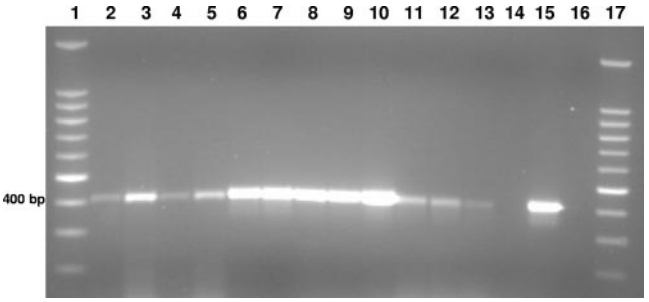

FIG. 1. Amplification of IS*900* sequences from plaque DNA. *M. avium* subsp. *paratuberculosis* strains ATCC 19851, 377 pw, B4, DVL 943, and *M. smegmatis* were processed through the FPTB assay. Plaque DNA was extracted from four plaques for each *M. avium* subsp. *paratuberculosis* strain and for *M. smegmatis*. Samples were then randomized, and PCR amplification of IS*900* was performed using primers P90 and P91. PCR products (30 μl) were analyzed by agarose gel electrophoresis (2% [wt/vol]). After PCR amplification, samples were decoded and grouped by sample type before gel analysis. Lanes: 1 and 17, 100-bp DNA size markers; 2 to 4, *M. avium* subsp. *paratuberculosis* ATCC 19851; 5 to 7, *M. avium* subsp. *paratuberculosis* B4; 8 to 10, *M. avium* subsp. *paratuberculosis* DVL 943; 11 to 13, *M. avium* subsp. *paratuberculosis* pw 377; 14, *M. smegmatis*; 15, *M. avium* subsp. *paratuberculosis* ATCC 19851 genomic DNA (positive control); 16, sterile distilled water (negative control). Expected band size for the P90 and P91 primers is 400 bp.

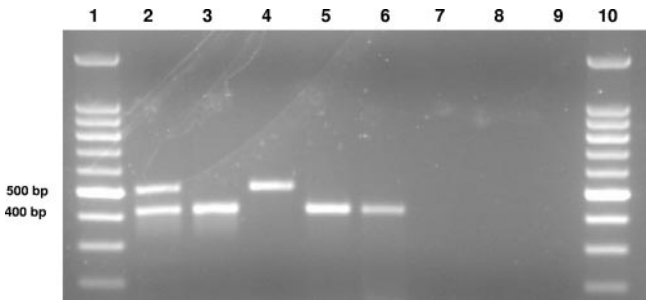

FIG. 2. Duplex PCR amplification of plaque DNA samples. Purified genomic DNA was used as a template to optimize duplex PCR conditions for the P90-P91 and JB21-JB22 primer pairs (lanes 2 to 4). For plaque samples, *M. avium* subsp. *paratuberculosis* strain ATCC 19851, *M. bovis* BCG, and *M. smegmatis* were processed through the FPTB assay. DNA was extracted from plaques for each strain, and duplex PCR amplification was performed (lanes 5 to 9). For mixed plaque samples, DNAs from two separate plaques were combined in one tube, and then 10-μl samples of the mixture were added to the PCR. Lanes 1 and 10, 100-bp DNA size marker; 2, *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG genomic DNA; 3, *M. avium* subsp. *paratuberculosis* genomic DNA; 4, *M. bovis* BCG genomic DNA; 5, mixed *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG plaque DNA; 6, *M. avium* subsp. *paratuberculosis* plaque DNA; 7, *M. bovis* BCG plaque DNA; 8, *M. smegmatis* plaque DNA; 9, sterile distilled water (negative control). Expected band sizes are 400 bp for the *M. avium* subsp. *paratuberculosis*-specific IS*900* sequence and 500 bp for the *M. bovis*-specific sequence.

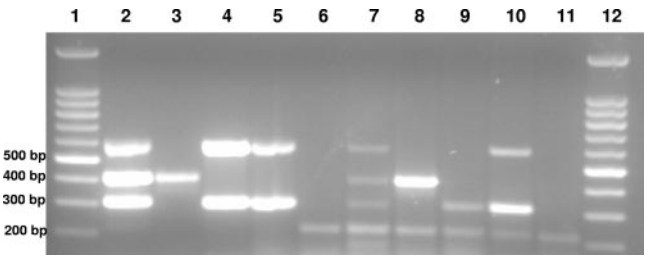

FIG. 3. Multiplex PCR amplification of *M. avium* subsp. *paratuberculosis* and TB complex sequences. Purified genomic DNA was used as a template to optimize the multiplex PCR conditions for the P90-P91 (IS*900*), TB850-TB284 (IS*6110*), and BW6-BW7 (IS*1081*) primer pairs (lanes 2 to 6). For mixed DNA samples, equal amounts of purified genomic DNA were mixed together and a sample (5 μl) of this used as template. For plaque samples, *M. avium* subsp. *paratuberculosis* strain ATCC 19851, *M. bovis* BCG, *M. tuberculosis*, and *M. smegmatis* were processed through the FPTB assay. DNA was extracted from plaques for each strain, and duplex PCR amplification was performed (lanes 6 to 9). For mixed plaque samples, DNAs from two separate plaques were combined in one tube, and then samples (10 μl) of the mixture were added to the PCR. Lanes: 1 and 12, 100-bp DNA size marker; 2, mixed *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG genomic DNA; 3, *M. avium* subsp. *paratuberculosis* genomic DNA; 4, *M. bovis* BCG genomic DNA; 5, *M. tuberculosis* genomic DNA; 6, *M. smegmatis* genomic DNA; 7, mixed *M. avium* subsp. *paratuberculosis* and *M. bovis* BCG plaque DNA; 8, *M. avium* subsp. *paratuberculosis* plaque DNA; 9, *M. bovis* BCG plaque DNA; 10, *M. tuberculosis* plaque DNA; 11, *M. smegmatis* plaque DNA. Expected band sizes are 400 bp (IS*900*), 550 bp (IS*6110*), and 306 bp (IS*1081*). The 200-bp band is generated by amplification of an uncharacterized sequence from the *M. smegmatis* genome by the BW6-BW7 primer pair.

losis progenitor cell within the plaque and was not due to any uninfected *M. avium* subsp. *paratuberculosis* cells present in the surrounding agar.

Specificity of the plaque PCR. A blind trial was performed to test the reproducibility and reliability of the IS*900* plaque PCR assay in that the identities of the samples being tested were not known until after the PCR results were available. The sample group consisted of 16 *M. avium* subsp. *paratuberculosis* plaques (4 plaque samples from 4 different *M. avium* subsp. *paratuberculosis* strains) and 4 plaques originating from *M. smegmatis* cells. The operator was blinded to the origins of the samples until after the results interpretation. A total of 20 plaques were tested by PCR for the presence of the IS*900* gene, and only the 16 plaque samples originating from *M. avium* subsp. *paratuberculosis* cells gave a positive PCR result. Besides this blind trial, when using the combine phage-PCR assay, we achieved figures of 100% reproducibility and 100% specificity overall ($n = 73$) (i.e., samples containing *M. avium* subsp. *paratuberculosis* cells were always plaque positive, and IS*900* PCR products were achieved only when *M. avium* subsp. *paratuberculosis* cells were present).

Differentiation of *M. avium* subsp. *paratuberculosis* from *M. tuberculosis* complex (TB complex). Having demonstrated that the target organism in the plaque can be identified by PCR, we next attempted to develop a duplex PCR to differentiate between *M. avium* subsp. *paratuberculosis* and *M. bovis*, since these two mycobacteria may also cause disease in cattle. For detection of *M. bovis*, primers JB21 and JB22, which target a single copy 500-bp gene fragment from *M. bovis* (21), were chosen. For detection of *M. avium* subsp. *paratuberculosis*, the IS*900* primers (P90 and P91) were used again.

Reaction conditions for duplex PCR were optimized using genomic DNA, and it was shown that the 400-bp IS*900* fragment from *M. avium* subsp. *paratuberculosis* ATCC 19851 and the 500-bp fragment from *M. bovis* BCG NCTC 5692 could be amplified, either individually or in combination (Fig. 2, lanes 2 to 4), showing no interference by either primer set. However, when *M. bovis* BCG plaque DNA was use as the template for the duplex PCR, no PCR product was generated, while the *M. avium* subsp. *paratuberculosis* plaque samples were PCR positive (Fig. 2, lanes 5 to 7). The failure of the JB21 and JB22 primers to amplify a single-copy gene from *M. bovis* BCG plaque DNA may indicate that single-copy genes are below the limit of the PCR identification step.

As an alternative strategy, a multiplex PCR based on multicopy insertion sequences from the TB complex was designed (Table 1). Using a combination of the three sets of primers (P90 and P91 for *M. avium* subsp. *paratuberculosis* [IS*900*], TB850 and TB284 [IS*6110* {27}], and BW6 and BW7 [IS*1081* {24}] for TB complex bacteria), multiplex PCRs were performed using either genomic DNA or plaque DNA (Fig. 3). The same PCR conditions were used as those optimized for the IS*900* PCR, but $MgCl_2$ concentrations were increased to 3 mM due to the higher primer concentrations. The multiplex PCR was able to amplify all three insertion sequence fragments from genomic DNA from the respective mycobacterial species with or without the presence of competing DNA from the other cell types (Fig. 3, lanes 2 to 5). No nonspecific PCR products were seen, indicating that nonspecific priming was not occurring when unrelated mycobacterial DNA was present (Fig. 3, lane 2). However, when genomic DNA from *M. smegmatis* was used as a template, a 200-bp fragment was amplified

(lane 6). Each primer pair was tested individually with the *M. smegmatis* genomic DNA template, and it was found that this band was seen only when using the IS*1081* primers (data not shown). BLAST analysis of the IS*1081* primers against the partially annotated *M. smegmatis* MC2 155 genomic sequence (TIGR) showed that primer BW6 could be aligned with a sequence at position 6962740 with a match of 21 out of 23 bases, suggesting that a mispriming is possible; however, no homology with the BW7 sequence primer was found. Sequence analysis of the amplified fragment did not show homology with any of the *M. smegmatis* sequence currently in the database; however, a complete genome sequence for this organism is not yet available, and the sequence amplified may be specific to the strain supplied in the FPTB kit. While we could not positively determine the origin of the 200-bp band, it fortuitously provides us with an internal positive control for the combined phage-multiplex PCR test, since *M. smegmatis* DNA is always present in plaque DNA samples.

The multiplex PCR was then carried out using *M. tuberculosis*, *M. bovis* BCG, and *M. avium* subsp. *paratuberculosis* plaque DNA either alone or in combination. The appropriately sized PCR products were amplified from *M. avium* subsp. *paratuberculosis* (400 bp), *M. bovis* BCG, and *M. tuberculosis* plaques (550 bp and 306 bp), respectively (Fig. 3, lanes 7 to 11). In addition, as expected, the 200-bp band was amplified from *M. smegmatis* DNA present in all DNA plaque samples. Only the 306-bp product from the IS*1081* element was amplified from *M. bovis* BCG plaque DNA (Fig. 3, lane 9). This is in contrast to the result using genomic DNA (Fig. 3, lane 4), where both the IS*1081* and IS*6110* bands were amplified. However, *M. bovis* BCG is known to contain only one to two copies of the IS*6110* element, and again this points to the fact that the PCR identification step is not able to reliably amplify a single-copy genomic sequence from the progenitor cell in a plaque from the FPTB assay. The 300-bp product was successfully amplified from the *M. tuberculosis* plaque, consistent with the fact that they are reported to contain 18 to 20 copies of the IS*6110* element (Fig. 3, lane 10).

Detection of *M. avium* subsp. *paratuberculosis* in naturally infected milk samples. To determine whether the assay could be used to detect *M. avium* subsp. *paratuberculosis* in milk recovered from naturally infected animals, a small trial was carried out with a herd which was confirmed to be infected with Johne's. Individual milk samples were collected from cows showing signs of weight loss and/or scouring and then chilled on ice before being transported to the laboratory. Bacterial cells were sedimented (2,500 × *g*, 15 min), the supernatant removed by aspiration, and the pellet resuspended in 1 ml of Media Plus before directly proceeding with the Phage amplification assay without any further decontamination steps. After overnight incubation, some growth of competitive microflora could be seen in the lawn, although this was not sufficient to obscure the development of plaques. Milk from 10 out of the 15 animals tested was plaque positive in the assay (Table 3). Plaques were then picked and DNA extracted for PCR analysis and in each case IS*900* was detected in some of the plaques. In general, those samples with the highest numbers of plaques had the highest percentages of IS*900*-positive samples. The fact that some plaques were not positive for IS*900* indicates that mycobacteria other than *M. avium* subsp. *paratuberculosis* were also present in the samples and demonstrates the requirement for the combined phage-PCR test to increase the specificity of the phage-based assay.

TABLE 3. Detection of *M. avium* subsp. *paratuberculosis* in naturally infected samples

Animal no.	No. of plaques recorded (PFU per 25 ml)[a]	IS*900* PCR amplification	
		No. of plaques tested	% Plaques positive[b]
1	16	21	9.5
2	5.5	8	25
3	1.5	9	56
4	20.5	4	25
5	5.5	9	22
6	1	2	50
7	0	ND[c]	
8	0	ND	
9	>100	2	100
10	>100	5	60
11	>50	3	67
12	0	ND	
13	7.5	10	50
14	0	ND	

[a] Average value from two 25-ml samples.
[b] Not all plaques gave a positive result with the IS*900* PCR. Tests were continued on individual samples until at least two positive tests were achieved to confirm the initial result. For sample 6, only two plaques were available for testing.
[c] ND, not done.

DISCUSSION

Studies of the physiology and ecology of *M. avium* subsp. *paratuberculosis* are limited by its fastidious nature and slow growth. When isolating the organism from samples containing a complex microflora, harsh decontamination methods are required to prevent overgrowth during the extended periods of incubation. Molecular tests, such as RT-PCR, can be used to detect *M. avium* subsp. *paratuberculosis* and offer live/dead differentiation but are also limited by the need to decontaminate and further purify samples to remove inhibitors of the PCR. This again leads to a loss of sensitivity and makes the assays both time consuming and complex. In this work we describe an adaptation of the FPTB assay for the detection of viable *M. avium* subsp. *paratuberculosis* cells. The assay is rapid and simple, detecting and identifying *M. avium* subsp. *paratuberculosis* in 48 h using standard laboratory reagents and equipment.

Here we have used four bovine *M. avium* subsp. *paratuberculosis* strains from different sources, including those from a natural infection, and in each case the FPTB assay was able to detect the presence of viable cells. In addition to the strains used in the development of this test, we have also shown that the sequence strain K10, originally isolated from bovine feces, can also be detected using this assay. It will be interesting to extend the range of *M. avium* subsp. *paratuberculosis* isolates tested to include ovine strains; however, given the fact that the broad host range of the phage is an essential feature of the test to amplify the phage using a fast-growing *Mycobacterium*, it seems likely that they too will be sensitive to the phage infection. Indeed, although the assay was developed for the detection of TB complex bacteria, other studies have previously

reported the use of the phage amplification assay to detect other pathogenic mycobacteria (2, 3).

This study is the first report that actiphage (D29) infects *M. avium* subsp. *paratuberculosis*. This result is in contrast to a previous report by Foley-Thomas et al. (8) that D29 did not form plaque on a lawn of freshly grown *M. avium* subsp. *paratuberculosis* K10 cells. The problem here may have been the slow rate of *M. avium* subsp. *paratuberculosis* lawn development, leading to a failure to form plaques rather than an inability of the phage to infect. When using the FPTB assay, only one round of phage replication is dependent on *M. avium* subsp. *paratuberculosis*, with subsequent rounds of replication occurring in the fast-growing *M. smegmatis* used to generate the lawn. Therefore, the FPTB assay could be used in studies of mycobacteriophage to determine the infection range with other slow-growing mycobacteria, circumventing the need to generate lawns of the host cell. This would be of particular relevance for difficult-to-culture mycobacteria, such as *Mycobacterium leprae*, and this assay could be used to determine if phage-based genetic tools (see reference 20) could be used in the study of such organisms.

Carrying out viable count measurements for *M. avium* subsp. *paratuberculosis* is problematic due to clumping of cells and contamination of samples during extended periods of incubation, and therefore, a quantitative comparison of plaque number and CFU was not carried out. However, based on an estimate of cell mass by optical density, the number of plaques recorded was always in the expected range, indicating that the phage infected all these strains with the same efficiency, but further work will be needed to determine whether this is true for all *M. avium* subsp. *paratuberculosis* strains. Comparison of the efficacy of the FPTB assay and culture techniques would be necessary to determine any difference in the detection of viable *M. avium* subsp. *paratuberculosis* by the two techniques. By using killed cells in the assay, we have demonstrated that only viable cells are detected. This is in accordance with the use of the FPTB assay to determine sensitivity to antibiotics (FPTB multidrug resistance assay), where cells that are killed by antibiotic treatment do not give rise to plaques (1, 7, 13). Taken together, this work suggests that this assay provides a rapid and simple way to quantify viable cell numbers.

In the development of this assay, we have used the IS*900* multicopy element for the PCR confirmation of *M. avium* subsp. *paratuberculosis* cells. While some controversy exists concerning the specificity of this as a signature sequence for *M. avium* subsp. *paratuberculosis*, it is still the most widely used molecular diagnostic test. In the future, amplification of the ISMAP*02* element may increase the specificity of this assay (22). The fact that these elements are present in multicopies is beneficial for the amplification of DNA from single-cell samples, where losses may occur during the DNA extraction process. Ultimately the development of more-efficient DNA extraction procedures or the incorporation of nested PCR procedures to increase sensitivity may be possible and allow the reproducible amplification of single-copy genes, such as F57 (5, 18). Originally the focus of this work was the identification of *M. avium* subsp. *paratuberculosis* alone. However, samples from infected animals, such as milk, could potentially contain more than one *Mycobacterium* species, most notably *M. bovis*. By combining the phage detection method with PCR amplification, the assay now gives both live/dead differentiation and genotypic identification of cells. This is particularly relevant to the detection of *M. avium* subsp. *paratuberculosis* in unpasteurized milk, where other pathogenic mycobacteria may also be found.

Development of the multiplex PCR allows the identity of the cell giving rise to the plaque to be established using only one PCR test (*M. avium* subsp. *paratuberculosis*/TB complex differentiation). The finding that the IS*6110* element was not amplified from *M. bovis* BCG was not surprising, since this strain is known to contain only one copy of the gene (12), and again supports our conclusion that amplification of single-copy genes is currently beyond the limit of detection for plaque DNA samples. Wild-type *M. bovis* strains are known to contain variable numbers of this element, with the majority of them having six; however, isolates of *M. bovis* with higher numbers of this element have been characterized (23). Therefore, amplification of both bands may be expected when testing clinical samples, and so the absence of the IS*6110* band cannot be used to differentiate between *M. bovis* and the rest of the TB complex. Of the four members of the TB complex, only *M. bovis* and *M. tuberculosis* would be expected to be isolated from bovine unpasteurized milk in the United Kingdom. The remaining members of the TB complex, *Mycobacterium microti* and *Mycobacterium africanum*, are isolated predominantly from rodents suffering from tuberculosis and human tuberculosis patients in Africa, respectively. Therefore, even though the level of molecular identification achieved is only within the TB complex, this is unlikely to be of practical significance within Europe or America. However, irrespective of the exact species of bacterium detected, the presence of TB complex bacteria in milk samples is likely to warrant further investigation of the herd. Hence, we have developed a generically useful test that can now be applied for the identification of either *M. avium* subsp. *paratuberculosis* or TB complex from milk samples, and further work is being undertaken to develop the test further and to carry out a full evaluation of its use both in the routine testing of milk samples and in clinical applications.

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